Partial deletion of chromosome 11p in breast cancer correlates with size of primary tumour and oestrogen receptor level

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Summary In a study of DNAs from 100 breast cancer patients and 100 controls, there were no differences in the frequencies of common or rare alleles at the Harvey ras (c-Ha-ras) locus on chromosome 11. However, one Ha-ras allele was deleted from the tumour DNA in 14 of 65 informative patients. Loss of a Ha-ras allele correlates with paucity of oestrogen receptor protein and with increased tumour size at presentation, but is not associated with microscopic evidence of lymph node invasion. The findings on Ha-ras and other informative loci are consistent with the possibility that a tumour suppressor gene involved in the early stages of breast cancer is located on the short arm of chromosome 11.

The human Ha-ras oncogene, homologous to the transforming sequence of the Harvey murine sarcoma virus, has been assigned to the short arm of chromosome 11 (McBride et al., 1982). On the 3' side of the gene lies a non-coding region made up of a variable number of repeated sub-units (Capon et al., 1983). Digestion with the restriction enzyme Bam Hl generates a restriction fragment length polymorphism with 4 common and several rarer alleles. Krontiris et al. (1985) have reported an increased frequency of rare alleles in patients with a variety of solid tumours and haematological malignancies; their series included a small number of breast cancers. This finding would imply that an inherited predisposition to cancer is linked to alleles at the Ha-ras locus. Several subsequent studies have sought to test Krontiris' hypothesis as applied to lung cancer (Heighway et al., 1986), myelodysplasia (Thein et al., 1986), colonic adenocarcinoma (Ceccherini-Nelli et al., 1987), familial melanoma (Gerhard et al., 1987; Hayward et al., 1988) and breast cancer (Lidereau et al., 1986). Only in the last two of these has supporting evidence been forthcoming (Lidereau et al., 1986; Hayward et al., 1988). However one group has recorded that a proportion of breast tumours, from patients constitutionally heterozygous at the Ha-ras locus, express only one allele or show a marked disparity in the intensity of the two allelic bands, suggesting that most or all of the tumour cells have undergone loss of a part of chromosome 11p (Theillet et al., 1986; Ali et al., 1987). Reduction to homo- (or hemi-) zygosity at specific genetic loci was recognised initially in retinoblastoma and subsequently in several other tumours (Knudson, 1971; 1985). The loci involved are believed to be sites of tumour suppressor genes or 'anti-oncogenes' which are relevant both to somatic events giving rise to sporadic tumours and to genetic predisposition to cancers (Lancet, 1988). In view of the potential importance of these issues for breast cancer screening programmes we have undertaken a survey of Ha-ras alleles in a cohort of 100 breast cancer patients.

Patients and methods

Tumour and venous blood samples have been collected from 100 consecutive patients with histologically proven breast cancer, prior to any treatment (apart from the anti-oestrogen tamoxifen). All patients had presented with palpable breast lumps and were referred by their general practitioners to the breast clinic in the Royal Infirmary of Edinburgh. Patients

with T4 tumours or with distant metastases at presentation, were excluded, as they are usually treated by chemotherapy in the first instance. The surgical procedures performed were either modified Patey mastectomy with axillary clearance, or wide local excision with axillary lymph node sampling. The resected specimen was immediately examined by the pathologist, tumour diameter measured in mm, and blocks taken for histological examination and for oestrogen receptor protein assay. The remainder was frozen on dry ice for later DNA extraction.

Lymph nodes were processed and examined for microscopic metastatic invasion. Tumours were classified into histological types as previously reported (Page & Anderson, 1988). Oestrogen receptor concentration was determined immediately by a saturation analytical method with separation of free and bound hormone using Dextran-coated charcoal adsorption as previously described (Hawkins et al., 1981). Samples from patients who had received Tamoxifen were rechecked by enzyme immunoassay (Leclercq et al., 1986). One hundred fresh placental samples have also been collected to act as a panel of normal controls representative of the local Edinburgh population. Permanent lymphoid cell lines were established from many of the blood samples by transformation in vitro with Epstein Barr virus. DNA was extracted from tumour and placental tissues and from blood and lymphoid cell lines (Steel, 1984). Ten μg aliquots of genomic DNA were digested to completion with Bam Hl (Roberts et al., 1977) (Boehringer, Mannheim GmbH), electrophoresed through 0.8% agarose, transferred to nylon membranes (Hybond, Amersham) and hybridised according to the manufacturer's instructions with the Harvey ras probe pEj (Shih & Weinberg, 1982), nick translated to a specific activity of $5 \times 10^7 - 1 \times 10^8 \, \text{cpm} \, \mu\text{g}^{-1}$ (Rigby et al., 1977). After hybridisation, filters were washed at 65°C with 0.1XSSC (15 mM Na Cl, 1.5 mM NA$_3$ citrate, 0.1% Na PPi, 0.1% SDS) and exposed to Kodak X-Ar film at −70°C for 7–14 days, with intensifying screens. Similar procedures were followed with other probes and restriction enzymes as tabulated below.

Results

Allelic frequency

The four major c-Ha-ras Bam Hl alleles A$_1$–A$_4$, together with one rare variant A$_1'$ are shown in Figure 1.

Table I shows the relative frequencies of these alleles in blood and/or lymphoid cell line DNA from 100 breast cancer patients and in DNA from 100 placentae.

There was no significant difference between breast cancer

Figure 1 Alleles of c-Ha-*ras* (Bam Hl digests) from four placental DNA samples. The left hand track of this Southern Blot contains a 'doublet' of allele A_1 and the rare variant A'_1.

Table I Bam Hl alleles of Harvey *ras* locus

	100 Breast cancer patients		100 Placentae	
	Number	%	Number	%
A_1	126	63.0	135	67.5
A_2	25	12.5	27	13.5
A_3	23	11.5	19	9.5
A_4	19	9.5	15	7.5
Rare alleles[a]	7	3.5	4	2.0

[a]A'_1 and A''_1 ... slightly smaller and larger respectively than A_1.

patients and controls in the frequencies of rare Harvey *ras* alleles; nor was there any shift in the distribution of common alleles between the two groups.

Allele loss in tumours

Complete or partial loss of a c-Ha-*ras* allele was established by comparing paired tumour DNA and white blood cell DNA samples from the same patients (Figure 2).

The one hundred tumours analysed fall into the following three categories. No allelic loss at the Ha-*ras* locus (51 tumours), loss of one allele (14 tumours) and uninformative, because the patient was constitutionally homozygous (35 tumours).

There was no preferential loss or retention of any of the four common alleles and our present analysis does not allow us to determine the maternal or paternal derivation of a deleted allele. We found no significant correlation between allelic loss and menopausal status, age, or history of an affected first degree relative. However, as shown in Table II, there was a significant correlation between loss of a Ha-*ras* allele and paucity of oestrogen receptor protein; absence of oestrogen receptor being a well-recognised index of poor prognosis (Croton *et al.*, 1981; Moore *et al.*, 1983; Williams *et al.*, 1987).
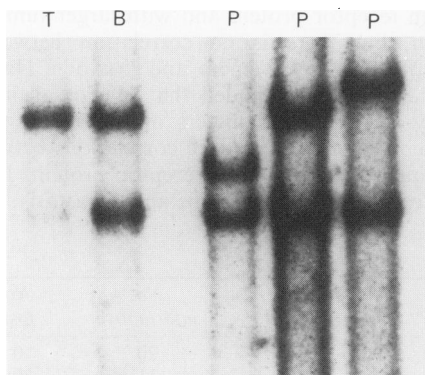
Figure 2 Bam Hl digests of tumour (T) and blood leukocyte (B) DNA from the same patient, probed with c-Ha-*ras* and compared with three placental controls. Note that alleles A_1 and A_3 are of equal intensity in B but allele A_1 is almost absent from the tumour sample.

There was also a significant correlation between tumour size and allelic loss as shown in Figure 3.

There was no significant correlation between allelic loss and pathological lymph node involvement, vascular invasion or histological type of tumour.

In order to assess the specificity of loss of the Harvey *ras* allele we have examined up to 5 other loci on the short arm of chromosome 11, comparing tumour DNA with lymphoblastoid cell line DNA from the same patient, as detailed in Table III.

Heterozygosity was found on a total of 49 occasions and the corresponding tumours had lost an allele in 19 cases (38.8%). Nineteen tumour/cell line pairs have been fully characterised for all 5 loci and allelic loss at one or more has been found in 10 (53%).

We have also studied one informative locus (pepsinogen) on the long arm of chromosome 11 and three at other chromosomal sites (5q, 6p and 17q). Of 67 instances where the patient was constitutionally heterozygous allele loss in the tumour was found outside of the 11p region on only one occasion (Table IV).

Discussion

These results, in agreement with several published studies (Krontiris *et al.*, 1985; Heighway *et al.*, 1986; Thein *et al.*, 1986; Ceccherini-Nelli *et al.*, 1987; Gerhard *et al.*, 1987) demonstrate that rare Ha-*ras* alleles can be identified in the normal population. We have found no evidence for an increased frequency of rare alleles in breast cancer patients, contradicting both Krontiris' initial report (Krontiris *et al.*, 1985) and the findings of a subsequent larger series (Lidereau *et al.*, 1986) in which rare alleles were identified in 41% of breast cancer patients. Heighway *et al.* (1986) reported a preponderance of the A4 allele in patients with non-small-cell lung carcinoma, but several other studies have failed to find evidence of linkage in myelodysplasia (Thein *et al.*, 1986), colorectal adenocarcinoma (Hayward *et al.*, 1988) or familial melanoma (Gerhard, 1987). Lidereau's study on breast cancer patients was performed on breast tumour material which had been stored for up to 7 years, while the controls were fresh blood samples from unaffected individuals. Wyllie *et al.* (1988) has suggested that prolonged storage could lead to the identification of spurious 'rare' alleles, and we have therefore used DNA from white blood cells or lymphoblastoid cell lines, as well as tumour material.

In contrast to these negative findings, the observation that a substantial proportion of breast cancers have lost one c-Ha-*ras* allele confirms the recent report of Theillet *et al.* (1986) and lends some support to the hypothesis that the Ha-*ras* locus may be involved in breast cancer, albeit on a rather different theoretical basis.

Knudson's 'two hit' hypothesis (Knudson, 1971) provides a link between the molecular mechanisms underlying familial and sporadic forms of the same type of cancer. In sporadic cancer, a cell undergoes a somatic mutation, which must then be followed by a second event to express the malignant phenotype, either a second somatic mutation or loss of the unmutated allele by non-disjunction or deletion.

Following the localisation of the retinoblastoma gene to 13q14 (Cavenee *et al.*, 1983) comparable deletions at other sites have been reported in a variety of tumours, including Wilms' tumour (Koufos *et al.*, 1985), lung cancer (Kok *et al.*, 1987) and acoustic neuroma/meningioma (Seizinger *et al.*, 1986).

The present findings raise the question 'Is the reduction to homozygosity of the Harvey *ras* gene in breast cancer merely an indication that there has been a deletion somewhere on chromosome 11 and is there another gene in the region much more directly involved in the disease?' Ali and colleagues (1987) recently reported a total of 14 allele losses, distributed between five polymorphic loci on 11p in breast

Table II   Relationship between loss of a c-Ha-ras allele and oestrogen receptor level in 61 breast tumours
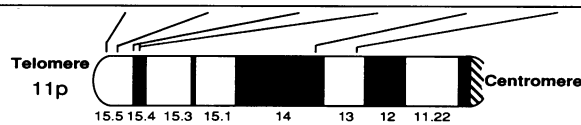
	ER poor/-ve $<20\,fmol\,mg^{-1}$ protein	ER moderate/rich $\geqslant 20\,fmol\,mg^{-1}$ protein (20)
Allelic loss	8	6
No allelic loss	10	37

$P = <0.02.$
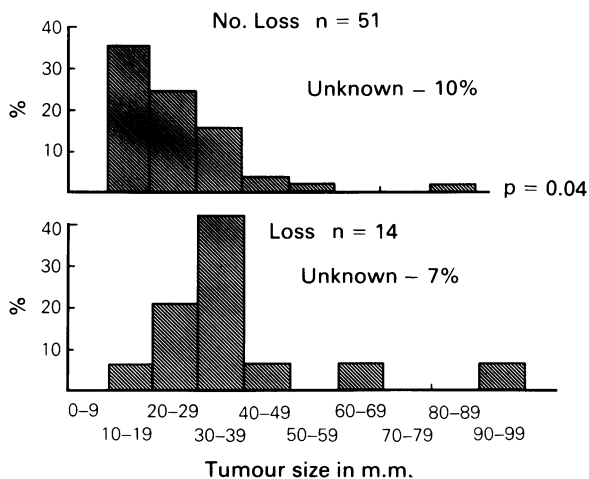
Figure 3   Distribution of primary tumour size (greatest diameter mm) in relation to Ha-ras allele status.

Table III   Details of allelic losses on 11p in breast tumour DNA compared with corresponding lymphoid cell line DNA

Tumour/ cell line pair	Ha-ras *1	β-globin *2	PTH *3	Calcit *4	FSH-β *5,6	P11F9 *7,8
1	a−	u	a−	a−	a−	a−
2	a−	a−	u	u	a−	u
3	a−	u	u	a−	u	u
4	a−	u	u	ab	ab	u
5	a−	u	u	u	u	ab
6	a−	u	u	u	u	ab
7	a−	ab	u	u	u	ab
8	a−	ab	a−	u	a−	ab
9	ab	u	u	u	u	a−
10	ab	u	u	u	u	ab
11	ab	ab	ab	u	u	ab
12	ab	ab	ab	u	u	ab
13	u	ab	u	u	ab	a−
14	u	ab	a−	u	u	a−
15	u	u	a−	u	u	a−
16	u	ab	u	u	u	ab
17	u	u	ab	u	ab	u
18	u	a−	u	u	u	ab
19	u	a−	u	u	u	ab

ab = informative, not lost; u = uninformative; a− = allele loss. 'a' and 'b' should not be taken to refer to specific alleles.
*1 = Shih & Weinberg (1982); *2 = Deisseroth et al. (1978); *3 = Naylor et al. (1982); *4 = Höppener et al. (1984); *5,6 = Glaser et al. (1985), Watkins et al. (1985); *7,8 = Porteous et al. (1987), Boyd et al. (submitted).

cancers from 9 patients, not all of whom were analysed for every locus. We find reduction to hemizygosity of several sequences other than Harvey ras on the same chromosome arm, at least one locus being involved in 10 of 19 tumours (53%), a frequency even higher than the corresponding figure for Ha-ras (21.5%) and certainly much higher than for informative loci outside 11p (1 of 67 informative loci, in 27 breast tumours). It might be unwise to extrapolate from the present data, for example, to suggest that 11p deletions can be inferred in almost 50% of primary breast cancers since only 19 tumours have been analysed in detail so far and they include 8 already known to have lost a Ha-ras allele. Nevertheless there has clearly been a substantial frequency of DNA lesions within the short arm of chromosome 11 in our tumour material. The simplest interpretation is that a single mitotic recombination event has caused loss of all loci distal to the breakpoint which, in some instances, must have been on the centromeric side of the most proximal sequence examined, P11F9, at 11p13. At least three of the tumours studied, however, show patterns of allele loss incompatible with this simple mechanism since one or more loci on the telomeric side of the region of hemizygotisation remain heterozygous and in one case (No. 8, Table III) there were two regions of hemizygotisation separated by a locus that remains heterozygous. It is necessary, therefore, to invoke either multiple mitotic recombination events, localised chromosome deletions, partial inversions or even more complex rearrangements. More extensive mapping studies are required to resolve the issues raised by these observations and analysis is now being extended to cover all one hundred tumours in our series. One objective is to identify the smallest region of chromosome 11p that is consistently included in any deletion that can be mapped. Such a region

might then be the site of a putative tumour suppressor gene (Friend et al., 1988).

This of course does not preclude the specific involvement of other regions of the genome not examined in the present study and it is quite possible that two or more putative anti-oncogenes are involved in breast cancer (Lancet editorial, 1988). It is relevant to note that loss of heterozygosity has been reported on 13q in 6 out of 10 ductal breast cancers (Lundberg et al., 1987).

We find that loss of a Ha-ras allele correlates with paucity of oestrogen receptor protein and with larger tumour size at presentation, but there is no correlation between pathological lymph node involvement and loss of a Ha-ras allele. Theillet et al. (1986) concluded that Ha-ras allelic loss was significantly linked to parameters of tumour aggressiveness since, in their material there were correlations between allelic loss and paucity of oestrogen receptor protein, histological grade and early occurrence of distant metastasis.

Table IV

Gene or probe	Designation	Localisation	Restriction enz	Ref[a]	No. examined	No. informative	No lost
Pepsinogen	pH PEP	11q 12	EcoR 1	1,2	24	20	0
Erb A₂	pHeA2	17q 21.3	Bam H1	3	20	11	0
MHC Class II	p11-B-4	6p 21.3	EcoR 1	4	21	21	0
λ MS 8	D8 S43	5q 34qter	Hin F1	5,6	18	15	1
				Total		67	1

[a]1,2 = Taggart et al. (1985; 1987); 3 = Gosden et al. (1986); 4 = Gustafsson et al. (1984); 5,6 = Solomon et al. (1987), Wong et al. (1987).

Of the six breast tumours identified in this series as uninformative or heterozygous for c-Ha-*ras*, but showing loss of an allele at one or more loci elsewhere on 11p (cases 9, 13, 14, 15, 18 and 19, Table III), five had low or absent oestrogen receptor. The correlation between this prognostic feature and hemizygotisation of sequences somewhere on 11p therefore does not seem to be exclusive to the Harvey-*ras* locus. Although such a conclusion must be tentative until a larger number of tumours has been analysed in similar detail, the suggestion is that c-Ha-*ras* serves as a relatively inefficient index of hemizygotisation of a specific locus some distance away.

It will be of great importance to map that putative locus and thereafter to reassess the clinico-pathological correlations already established, to see if information of prognostic value can be obtained from DNA analysis of tumours at presentation. The clinical relevance of such findings will be established by follow up of our patient cohort to gather data on disease-free interval and long-term survival. Ultimately the objective is to define the gene itself and to establish the mechanism whereby it contributes to the evolution of breast cancer.

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